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Training School

Genetics and Agronomy for Low Acrylamide-Forming Potential in Cereals

22 – 24 May, 2024

Maize Research Institute, Zemun Polje
Belgrade, Serbia

Edited by Slađana Žilić
May, 2024

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ACRYRED

ACRYRED • COST Action CA21149
Reducing Acrylamide Exposure of Consumers by a Cereals Supply-chain Approach Targeting Asparagine

TRAINING SCHOOL

Genetics and Agronomy for Low Acrylamide-Forming Potential in Cereals

Maize Research Institute, Zemun Polje
Belgrade-Zemun, (SRB)

22nd to 24th of May 2024

Objectives:

The main aim of the Training School is to improve capacity on current knowledge in cereal molecular biology, breeding and agronomy targeting free asparagine in cereal genotypes through interdisciplinary research networking and knowledge exchange. The lectures and practical sessions in application of breeding, agronomy, molecular techniques and chemical methods for low-asparagine cereal genotypes identification and creation will be given.

Our lecturers:

Dr. Viktor Korzun (DE)
Dr. Elena Baldoni (IT)
Dr. Navneet Kaur (GB)
Dr. Valentina Španić (HR)
Dr. Khaoula El Hassouni (DE)
Dr. Marco Napoli (IT)
Dr. Natalija Kravić (SRB)
Dr. Ana Nikolić (SRB)
Dr. Marija Kostadinović (SRB)
MSc. Maša Buđen (SRB)
BSc. Snežana Savčić-Petrić (SRB)
Dr. Saša Janković (SRB)

Our trainers:

Dr. Ana Nikolić
Dr. Marijana Simić
Dr. Valentina Nikolić
Dr. Vesna Kandić Raftery
Dr. Manja Božić
MSc. Beka Sarić
Dr. Slađana Žilić
MSc. Maša Buđen
Jelena Škorić Sekulić
MSc. Dušan Urošević

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MAIZE RESEARCH INSTITUTE ZEMUN POLJE BELGRADE - ZEMUN

TRAINING SCHOOL

Genetics and Agronomy for Low Acrylamide-Forming Potential in Cereals
Date: May 22 – 24, 2024
Hosting Institution: Maize Research Institute, Zemun Polje (MRIZP) mrizp
Location: Belgrade-Zemun, Serbia

What is the main mission of the training school in CA 21149?

Interdisciplinary training, networking, and knowledge exchange to build capacity on monitoring and assessment of acrylamide mitigation measures applied in industrial production.

Program of the CA 21149 Training School

	1st Day, May 22nd (Wednesday)	
9:00-9:30	Registration	
9:30-9:40	Opening and welcoming remarks	Dr. Elena Baldoni (IT) Dr. Viktor Korzun (DE) Dr. Slađana Žilić (SRB)
9:40-9:45	Welcoming remarks in front of LO	Dr. Violeta Anđelković (SRB)
9:45-10:30	Genomic and molecular breeding in small-grain cereals	Dr. Viktor Korzun (DE)
10:30-11:00	Coffee break	
11:00-11:30	Plant genetic resources - Gene bank as source of a high quality cereal genotypes	Dr. Natalija Kravić (SRB)
11:30-12:15	Genome-wide association study (GWAS) in durum wheat for free asparagine content	Dr. Elena Baldoni (IT)
12:15-13:00	Field trialling of CRISPR low-asparagine wheat and investigation of the increase in asparagine in response to pathogen infection in wheat.	Dr. Navneet Kaur (GB)
13:00-14:00	Lunch break	
14:00-14:45	Indirect selection for lower mycotoxin accumulation in winter wheat grains	Dr. Valentina Španić (HR)

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14:45-15:30	Application of molecular biology techniques for identification and expression profiling of asparagine synthetase genes in cereals	Dr. Ana Nikolić (SRB)
15:30-15:45	Summery and conclusion of the day	Dr. Viktor Korzun (DE) Dr. Elena Baldoni (IT) Dr. Slađana Žilić (SRB)
16:00-18:00	Panoramic sightseeing of Belgrade (from the Institute)	

	2nd Day, May 23rd (Thursday)	
9:30-10:15	Options for reducing acrylamide precursor levels in the wheat supply chain include cultivar selection, cropping practices, milling procedures puffing and baking approaches.	Dr. Khaoula El Hassouni and Prof. Friedrich Longin (DE)
10:15-10:45	Coffee break	
10:45-11:30	Marker-assisted selection for high quality and safe maize.	Dr. Marija Kostadinović (SRB)
11:30-12:15	Impact of biotic and abiotic stressors on free asparagine formation in small-grain cereals and maize: Exploring correlations with grain quality and agronomic practices.	Dr. Marco Napoli (IT)
12:15-13:30	*Practical session Ia: PCR analysis of asparagine synthetase genes- setting up and performing qualitative and quantitative PCR (qPCR) reaction*	Dr. Ana Nikolić (SRB) Dr. Manja Božić (SRB)
12:15-13:30	*Practical session II: HPLC demo of asparagine determination.*	Beka Sarić (SRB) Dr. Slađana Žilić (SRB)
12:15-13:30	*Practical session III: color analysis of cereal-based food products by Konica Minolta.*	Dr. Marijana Simić (SRB) Dr. Valentina Nikolić (SRB)
13:30-14:30	Lunch break	
14:30-15:15	*Practical session Ib: PCR analysis of asparagine synthetase genes- monitoring of PCR reaction progress*	Dr. Ana Nikolić (SRB) Dr. Manja Božić (SRB)
15:15:15:45	Feedback of trainees / Open discussions	All
15:45-16:00	Summery and conclusion of the day	Dr. Elena Baldoni (IT) Dr. Viktor Korzun (DE) Dr. Slađana Žilić (SRB)
19:00-21:30	Dinner (Bohemian quarter Skadarlija)	

	3rd Day, May 24th (Friday)	
9:30-10:00	Usage of unmanned aerial vehicle (UAVs) in yield and quality prediction and cereal crop monitoring.	MSc. Maša Buđan (SRB)

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10:00-10:30	Acrylamide – State of play in Serbia (regulatory compliance with the EU and control of the food on the market)	MSc. Snežana Savčić-Petrić (SRB)
10:30-11:00	Coffee break	
11:00-11:20	Dietary acrylamide exposure in different Serbian population categories	Dr. Saša Janković (SRB)
11:20-12:40	*Practical session IV: Field visiting / demo of cereal crosses (fertilization)*	Jelena Škorić Sekulić (SRB) MSc. Dušan Urošević (SRB)
11:20-12:40	*Practical session V: Field visiting / Drone imaging of sulfur treated field*	MSc. Maša Buđan (SRB)
11:20-12:40	*Practical session VI: Field visiting / observation (evaluation of quality parameters of cereal plant)*	Dr. Vesna Kandić (SRB)
12:40-13:40	Lunch break	
13:40-14:00	Debrief, Certificates	Dr. Elena Baldoni (IT) Dr. Viktor Korzun (DE)
14:00-14:20	Closing remarks	Dr. Elena Baldoni (IT) Dr. Viktor Korzun (DE)
14:20-14:30	Farewell words	Dr. Slađana Žilić (SRB) Dr. Violeta Anđelković (SRB)
14:30-15:30	*Tour of the ZP Institute's laboratories and fields (facultative)*	Dr. Marijana Simić (SRB) Dr. Jovan Pavlov (SRB)

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ORAL PRESENTATIONS

(Invited lecturers)

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L I

Genomic and molecular breeding in small-grain cereals

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Feeding a global population set to reach 9 billion by 2050, in the face of climate change and pressure on finite natural resources, will require a 70% increase in global agricultural output while reducing input use and GHG emissions. At the same time, there are growing opportunities and demands for the use of plant-derived biomass to provide livestock feed and for other uses, including renewable materials to support the bioeconomy, thereby decreasing dependency on imports and reducing regional and global environmental impact.

Securing a sustainable global supply of high-quality food and feed and supplying renewable biomass for the production of bio-based products and energy are key priorities for both European and global societies. In the coming decades, the European plant sector will play a central and essential role in meeting this challenge and achieving this will depend strongly on the generation and transfer of new knowledge, and greater innovation in the plant sector.

In this context, genomics and especially related molecular genetic technologies play an important role in the creation of new plant varieties that optimally combine high and stable yields with resistance to abiotic stresses and biotic factors of the cultivation environment. Over the past decade, molecular marker technology has provided a wide range of innovative approaches to improve the efficiency of modern breeding strategies and methods. The availability of new molecular tools and technologies has a significant impact on the planning and development of the critical elements of breeding required to accelerate this time-consuming and laborious process. Monitoring of genetic diversity associated with successful breeding, targeted use of plant genetic resources, examples of specific applications of molecular markers in cereal breeding, the potential of genomic selection, and the use of genomics and gene edits in cereal breeding will be presented and discussed using the example of cereals.

Keywords: ACRYRED COST ACTION, breeding, wheat, grain quality, cereals

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L II

Gene bank as source of a high quality cereal genotypes

Natalija Kravić*, **Violeta Andjelković, Vojka Babić**
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According to the United Nations (UN), the world's population will reach 9.7 billion by 2050, which indicates the trends of increase in food, feed and other agricultural products demand for 41–51%, as well as the trends of increase in calorie demand [1]. Globally, ~29.3% of the population (i.e. 2.3 billion people) experience moderate-to-severe food insecurity. In addition, projections have shown that, by 2030, 670 million people will still be undernourished, representing ~8% of the population. All these reports clearly indicate that the world will need more food in the next 2.5 decades [2,3]. Furthermore, today's agricultural sector is facing a double challenge: on the one hand, to address the societal demand for more environmental-friendly practices, and diversified and healthier products, and, on the other, to sustain production challenged by climate change. The combination of both raises unprecedented questions regarding the identification and improvement of suitable plant material to deliver varieties to the farmers and other end users.

Cereals are the major crops that feed the world and the main pillar of food security worldwide. Primarily belonging to the Gramineae family, cereals include wheat, rye, barley, oats, rice, millet, maize, sorghum, and triticale, and represent important sources of lipids, carbohydrates, protein, minerals, and vitamins. Although being lower in protein, cereals (including maize, millet, and rice) are a major source of dietary protein in Mesoamerica and sub-Saharan Africa, West Africa, and Asia. In recent studies, it has been reported that the global potential for wheat, maize, and rice is 7.7, 10.4, and 8.5 t ha^{-1}, while their actual yields are 4.1 (53.24% from the full potential for wheat), 5.5 (52.88% from the full potential for maize), and 4.0 (47.05% from the full potential for rise) t ha^{-1}, respectively [4–6].

Enhancing genetic potential and narrowing the gap in crop yields is the most effective way to introduce the innovation needed in agriculture to meet the UN Sustainable Development Goals (SDGs), particularly zero hunger (SDG2), good health and well-being (SDG3) and life on land (SDG15). Namely, the UN SDG Target 2.5 explicitly calls for the maintenance of genetic diversity of seeds through soundly managed and diversified gene banks at national, regional, and international levels as an essential undertaking to end global hunger (SDG Goal 2).

Sustainably feeding the world's population by 2050 requires strategies to reduce and close the yield gap in cereals as priority crops. The current yield potential of major cereal crops is the result of multi-level efforts in classical breeding, advanced agronomic practices, and genetic engineering, while improvement in the productivity of crop plants requires the identification of traits that should be targeted for manipulation. The yield-determining traits could be grouped according to growth and development [7,8], as well as grouped according to interactions with the environment and other organisms [9–11]. The variation in these traits can be largely found in a rich reservoir of diversity maintained in *ex-situ* collections – gene banks and must be linked to a process for rapidly incorporating into new climate-smart gene-pools to be used for direct cultivation or as parents in (pre) breeding programmes targeting desirable traits [12]. Hence, in the context of food security, the principal aim is to update the evidence and enhance the understanding of the role and value of gene banks in a changing agricultural context. Gene banks perform a broad range of functions, from collecting new plant materials to conserving them in medium- and long-term storage, testing seed health, conducting

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research to characterize and evaluate the resilience of plant materials to abiotic and biotic stresses, and distributing plant materials to plant breeders, research scientists, farmers and food industry [13].

Cereal genetic resources maintained in *ex situ* collections – gene banks, are part of the world's vast biodiversity and the raw materials on which humankind relies for food, nutrition and livelihood security, and to support the bio-economy. The vast range of traits expressed in cereal genetic resources, and their adaptive capacity, are essential for enhancing the resilience of agricultural production systems, as well as for supporting advancements towards innovative, efficient agro-food systems and other bio-based value chains. Therefore, they are a key form of natural capital needed for stability and adaptability in agriculture and for a sustainable bio-economy. The underlying genetic diversity in the *ex situ* collections of cereals is the lifeblood of plant breeding, making conservation of the diversity of major crops critical, and mining these collections for useful traits [14,15]. The majority of cereal *ex situ* collections are securely preserved, but largely unused (less than 5% use). Even though cereal *ex situ* collections have increased over the last few decades in a global effort to conserve plant genetic resources, the size of these collections complicates the maintenance and evaluation of the genetic diversity they house [16]. The major obstacle to enhance germplasm accessions is the lack of adequate evaluation data, and thus, the inability to adequately respond to inquiries for those particular accessions that directly meet the user needs [17]. For the majority of them, only basic passport data (an internationally accepted set of data used to provide minimum necessary information on the accessions in *ex situ* collections) is available and data on unique proprieties/traits is generally lacking [18,19]. It has been stated that there are considerable gaps in basic documentation and characterization data for cereal genetic resources, and this is a major limitation for their utilization in breeding programmes. Although, as a whole, cereal *ex situ* collections have been characterized and evaluated, but on average, only 64% of them are characterized morphologically, 51% agronomically, 14% biochemically, 14% for abiotic traits, and 22% for biotic traits. Hence, the comprehensive characterization of cereal genetic resources is critical to add value to accessions and to further help guide users on selection of appropriate germplasm for their specific needs [20,21].

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L III

Genome-wide association study (GWAS) in durum wheat for free asparagine content

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Acrylamide, a neurotoxic molecule that is also suspected to be carcinogenic, has been detected in many foods after high-temperature processing. In wheat derivatives, free asparagine is a precursor of acrylamide formation. Increased levels may drive acrylamide quantity[1]. Therefore, the control of free asparagine levels in wheat seeds is of considerable interest in contemporary crop and food sciences. Currently, few information is available about the genetic control of this trait[2]. Our study aims to explore the natural variation of free asparagine levels in durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.)) grains to identify candidate genes controlling this trait. To this purpose, 201 durum wheat genotypes were selected from an international genotyped collection[3]. These genotypes were sown in an experimental field located in Italy for three years. Wholemeal flour was obtained from harvested grains and free asparagine content was measured using an enzymatic method. A good variability of this trait was shown. Both single-locus and multi-locus genome-wide association studies were performed to identify genomic regions associated with free asparagine content. Six Quantitative Trait Nucleotides on chromosomes 6A, 7A, 2B, 4B, 7B associated with this trait were identified. To deeply characterize metabolite variations associated to asparagine content, the whole grain metabolome of the 201 durum wheat genotypes was investigated by Ultrahigh Pressure Liquid Chromatography coupled to High Resolution Mass Spectrometry (UPLC-HRMS)[4]. Moreover, 100 selected samples were analysed through High Resolution Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy. Both spectroscopic determinations showed a good correlation with the enzymatic method for free asparagine content quantification, confirming the good variability observed in our experimental set. These analyses will allow to identify metabolic pathways associated to free asparagine accumulation and to characterize the metabolite composition of the whole grain of the 201 durum wheat genotypes.

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L IV

Field trialling of CRISPR low-asparagine wheat and investigation of the increase in asparagine in response to pathogen infection in wheat.

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In many plant species, asparagine plays a central role in nitrogen storage and transport. However, it also acts as a precursor for acrylamide, a Class 2A carcinogen that forms from free (soluble, non-protein) asparagine and reducing sugars during high-temperature cooking and processing of grains, tubers, beans, storage roots and other plant products. The accumulation of free (soluble, non-protein) asparagine increases during seed germination and in response to abiotic/biotic stresses.

Employing CRISPR/Cas9, we targeted the asparagine synthetase-1 and -2 genes (*TaASN1* and *TaASN2*) in bread wheat. The developed lines, assessed in field trials, exhibited significant reductions in free asparagine concentration, leading to a concurrent decrease in acrylamide formation in heated flour (Raffan et al., 2023) and baked products. The latest results from the trials will be presented.

We are also investigating the signalling proteins/transcription factors responsible for the accumulation of free asparagine under biotic stress, which currently remain unidentified. In wheat, fusarium head blight disease, caused *by F. graminearum* (*Fg*), reduces yield, and contaminates grains with mycotoxins, such as DON. DON treatment in wheat increases free asparagine, glutamine, and aspartate concentration (Warth et al., 2015). We aim to unravel this intricate signalling network by utilizing Fg mutants, RNA-seq analysis, phosphoproteomics and proximity labelling, then validate our signaling hub model using VIGS.

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L V

Indirect selection for lower mycotoxin accumulation in winter wheat grains

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Fungal pathogens are causing diseases of wheat that contribute to 15–20% yield losses per annum [1]. *Fusarium* spp. occur regularly every year on cereal crops around the world, and additional concerns have created new insight into the extremely negative effects of mycotoxins on human and animal health. Fusarium head blight (FHB), mainly caused by *Fusarium graminearum* and *F. culmorum*, can significantly reduce grain yield, seed quality and produces the mycotoxins that affects food safety [2]. Trichothecenes (nivalenol (NIV), deoxynivalenol (DON), fumonisins, T-2 and HT-2 toxins), and zearalenone (ZEN) and fumonisins are the major *Fusarium* mycotoxins occurring on a worldwide basis in cereal grains, animal feeds and forages [3]. Today, the term "food safety" is increasingly mentioned and hence mycotoxins are increasingly attracting attention, and scientists and breeders are working on solutions to find resistance wheat genotypes to this widespread disease. FHB continues to threaten susceptible wheat varieties where environmental conditions such as high moisture and temperature persist during the flowering period. Warm and humid environmental conditions ideally propagate the pathogen which may result in severe disease outbreaks with substantial crop losses. This especially attracts attention in time of enhanced climate changes. Typical FHB symptoms are water soaked lesions on the glumes, followed by discoloration that spreads from the point of infection to the neighbouring spikelets. As infection progresses, symptoms of wilting and blight spread through the entire head indicating premature senescence of infected heads [4].
Screening and identifying FHB resistant genes in wheat germplasm for development of resistant wheat varieties in combination with usage of cultural practices and fungicides are the most effective ways to manage FHB. But however, agronomical practice and fungicides may only partially reduce the risk of damage. The best applications of fungicide, considering timing and dose of application, may reduce FHB symptoms and resulting losses up to 70-80% [5]. Furthermore, FHB resistance has been classified into multiple types (Fig.1.). Type I resistance is attributed to reducing initial infection, type II resistance prevents spread of infection within the spike and type III confers resistance to mycotoxin accumulation. Type II resistance has been widely used in breeding programs due to its effective performance in reducing FHB impact on grain production. There is also evidence that FHB resistant wheat genotypes accumulate far less DON than susceptible ones [6].
Limits for some mycotoxins have been recommended and specified in unprocessed cereals, milling products, and cereal end-use products: 200–1750 μg kg^{-1} for DON, 20–400 μg kg^{-1} for ZEN, 200–4000 μg kg^{-1} for the sum of B1 + B2 fumonisins (FB1 + FB2 combined), and 15–1000 μg kg^{-1} for the sum of HT-2 and T-2 toxins. European Commission has not yet given any legislative for NIV, but the European Food Safety Authority set a tolerated daily intake (TDI) of up to 1.2 μg kg body weight per day [7]. Further, *Fusarium* mycotoxins and metabolites can be changed in their chemical structure, with unexpectedly high toxicity in the digestive tract of humans/animals although the metabolic fate is still not very well studied and they are not regulated. As the impact of many mycotoxins and metabolites is still unknown, it is very important to investigate their toxicity, and consequently regulate their concentrations in food and feed [8]. Also, very important task of the wheat breeders is to work on genetic resistance that is the most economical, environment-friendly and effective strategy to control

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FHB. It is believed that breeding for resistance to FHB will increase resistance to mycotoxin accumulation in most cases.

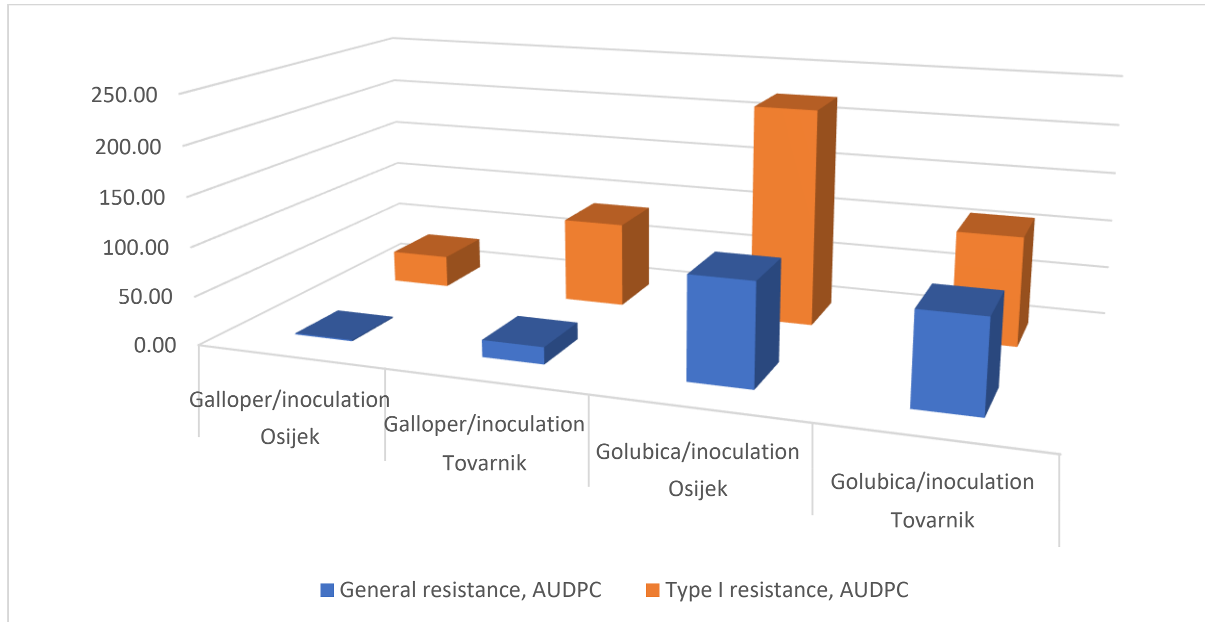

Fig.1. Area under disease progress curve (AUDPC) of two wheat varieties for general and Type I resistance in FHB inoculated treatment at two locations (Osijek and Tovarnik)

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L VI

Application of molecular biology techniques for identification and expression profiling of asparagine synthetase genes in cereals

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The enzyme asparagine synthetase catalyzes the transfer of an amino group from glutamine to aspartate, leading to the creation of glutamate and asparagine. A surplus of free (nonprotein) asparagine in crops poses a concern for food safety since it is the precursor of acrylamide, a potentially harmful contaminant formed during the processing of foods at high temperatures. Acrylamide has been categorized as a probable group 2A carcinogen by the International Agency for Research on Cancer (IARC) and is known to exhibit reproductive and neurotoxic effects at elevated levels [1].

Different approaches have been applied to reduce acrylamide forming potential in cereals, and the problem is still insufficiently explored from an agronomic and genetic perspective. The application of the results of these approaches could enable the food production processes to be modified without incurring high costs to comply with tightening regulations on acrylamide content [2].

Development of cereal varieties with reduced acrylamide forming potential requires detailed knowledge of asparagine metabolism genetic control. Genes, enzymes, metabolites, and environmental factors connected to asparagine metabolism have been described in a complex network, but most attention has been devoted to the enzyme at the center of that network, glutamine-dependent asparagine synthetase [3]. Therefore, of the nine enzymes involved in asparagine metabolism, asparagine synthase was the most frequently targeted by genetic interventions [2]. The family of asparagine synthetase genes has been extensively studied in the Poaceae family (*Triticeae*, *Brachypodiae*, *Panicoideae* and *Ehrhartoideae*) [1] and it is comprised of five genes per genome (per A, B, D genome) in *Triticeae* species with the genes assigned to four groups: 1, 2, 3 (3.1 and 3.2) and 4. It has been shown that some hexaploid common wheat and tetraploid emmer wheat lack a group 2 gene in the B genome (*TaASN – B2*). A phylogenetic analysis revealed that genes from the other studied species clustered with the *Triticeae* genes, whereas a group 2 gene is absent from brachypodium, sorghum, and maize. Rice has only one group 3 and one group 4 genes.

The presence/absence and expression level of these asparagine synthetase genes in different tissues and developmental stages was the subject in many studies on different *Poaceae* family species ([4], [5] and [6]) especially in Triticae tribe ([7], [8] and [9]). These analyses were conducted using both qualitative and quantitative PCR (*Polymerase Chain Reaction*) molecular biology techniques. The PCR technique is based on the enzymatic replication of DNA - a short segment of DNA is amplified using primer mediated enzymes. The enyzme DNA polymerase synthesises new strands of DNA complementary to the template DNA [10]. As the term implies, a qualitative PCR involves the use of polymerase chain reaction in order to determine the presence or absence of a specific DNA product. Further, quantitative reverse transcription polymerase chain reaction (RT-qPCR) is used to detect and quantify RNA. Transcribing total RNA into complementary DNA (cDNA) is the first step in the process. The cDNA is then used as the template for the quantitative PCR (qPCR) or real-time PCR reaction [11]. Using fluorescence, qPCR measures the amount of amplification product produced in each PCR cycle.

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RT-qPCR is used in gene expression analysis. Besides these two relatively simple techniques, more complex biotechnology approaches for creating cereal genotypes with low free asparagine level were applied. One of those approaches is application of gene editing. It is a technique that involves the process of making targeted modifications to the DNA of a cell or organism. It can be used to add, remove or alter DNA in the genome [12].

Studies examining the presence or absence of asparagine synthetase genes could assist in the selection of varieties with lower levels of free asparagine, due to the discovery of certain genes for this enzyme (*TaASN-B2*) being absent in them. Evidence suggests that the expression of 2 group genes in wheat (*TaASN2*) is most pronounced in the grain, with their expression in the embryo likely playing a pivotal role in regulating free asparagine concentrations in the grain as a whole. In addition, these findings uphold the argument that the natural deletion of the specified gene could be a valuable genetic variant for wheat breeders to exploit in order to decrease the free asparagine content in the grain [9]. Furthermore, the implementation of biotechnology methods proved to be highly effective in achieving this objective. Recent research has employed the CRISPR/Cas9 system to manipulate the *TaASN2* genes of wheat, resulting in partial or total gene knockouts. This genome editing technique has effectively reduced the concentration of free asparagine in the wheat [13]. All of these results point to molecular biology techniques as a powerful tool for uncovering solutions to the discussed issue in the future.

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L VII

Options for reducing acrylamide precursor levels in the wheat supply chain include cultivar selection, cropping practices, milling procedures puffing and baking approaches.

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Acrylamid is a clear carcinogenic compound that can be generated during the baking process of starchy products. It forms mainly from reducing sugars and free asparagine through the Maillard reaction. Acrylamid can be found in many food products especially in bread. Currently, the European commission is actively working on developing new guidelines for permissible acrylamide levels in various products to ensure food safety and minimize potential health risks. Our objective was to investigate how the acrylamide risk could be mitigated along the wheat supply chain. For this purpose, we conducted a series of different experimental investigations. Firstly, we run extensive baking trials and were able to clearly demonstrate that the free asparagine content in whole grain flour correlates very closely with the acrylamide content in bread. A long dough fermentation time can significantly reduce the acrylamide content in bread. Secondly, we demonstrated that acrylamide content in puffed durum grains highly correlated with free asparagine content in the whole grain flour of the durum grains. Finally, we performed a series of experiments to see how free asparagine content can be influenced by breeding, fertilization, and milling. Thereby, the levels of free asparagine in the flour varied greatly between the cultivars within the different tested species bread wheat, durum wheat, spelt, emmer and einkorn. Interestingly, free asparagine contents of the cultivars did not correlate with other quality traits such as protein content, sedimentation value, grain size and falling number. For fertilisation, it was found that increased nitrogen fertilisation led to higher free asparagine contents, but the choice of cultivar had a greater influence on free asparagine contents than fertilisation. Finally for milling, the more of the outer layers of grains are used for flour production, the higher was the content of free asparagine. Consequently, there are numerous options available along the wheat supply value chain to sustainably reduce the acrylamide risk in grain products. It is essential to consider, however, that only a quite small part of wheat products have acrylamide values higher than expected EU threshold values and, thus, only a small part of the wheat supply chain might need to work on acrylamide reduction strategies. Plant breeding has the potential to significantly reduce the acrylamide content in end products by developing cultivars with reduced asparagine content. Genome-wide association mapping using a bread wheat panel revealed a complex genetic architecture of asparagine content built up of few small quantitative trait loci (QTL). The largest QTL was located on chromosome 7B explaining 18% of the genotypic variance. The QTL alleles for low asparagine can then be pyramided via markers assisted selection and genetically driven crossing schemes to derive low asparagine cultivars for a reduced acrylamide in wheat end-products.

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L VIII

Marker-assisted selection for high quality and safe maize

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Molecular markers offer several advantages over the conventional phenotypic markers enabling quick and precise plant breeding and early generation selection for significant traits without extensive field research [1]. When molecular markers are tightly linked to genes of interest, they can be used to select indirectly for the desirable allele, what is the simplest form of marker assisted selection (MAS). The main advantages of MAS are direct selection of target gene with specific markers (foreground selection) and fast recovery of recurrent parent's genome (background selection). In foreground selection, homozygous and heterozygous plants can be distinguished with gene-specific markers. This enables maintenance of recessive genes without the need for progeny testing in each generation of selection. During backcrossing, molecular markers can help in identification of the genotypes with the highest proportion of recurrent parent's genome. Both foreground and background selection decrease the number of the generations required to create desirable genotype through conventional breeding based on the phenotypic selection [2].

The greatest progress in MAS has been achieved with the qualitative traits that are regulated by the action of a single or several genes and are clearly phenotypically defined. One successful example of MAS for maize quality improvement is the utilization of *opaque2*-specific SSR markers phi057 and umc1066 for development of quality protein maize (QPM) lines with enhanced nutritional quality [3]. Another example is the use of *lpa2-2*-specific SSR marker umc2230 for obtaining the agronomically superior lines with low phytic acid content [4]. Similarly, significant enhancement of kernel β-carotene in elite maize genotypes is being accomplished with the use of *crtRB1*-specific SSR marker for foreground selection [5].

Quality protein maize has high content of essential amino acids lysine and tryptophan that are deficient in standard maize kernel. Naturally occurring *opaque2* recessive mutation changes the protein composition of the maize endosperm, resulting in 2-3 fold higher concentrations of lysine and tryptophan [6]. Although QPM was developed primarily for human consumption where maize is staple food, its use in feeding is expected to decrease/remove the need for costly synthetic lysine and soybean as well as to improve livestock performances. Due to its higher biological value (amount of nitrogen that is retained in the body), balanced nitrogen index and leucine-isoleucine ratio, QPM offers significant nutritional benefits, which is well demonstrated both in terms of human food and animal feed [7].

Maize Research Institute Zemun Polje has a breeding program aimed to create QPM and β-carotene enriched genotypes adapted to temperate climate, using the integrated conventional and molecular breeding approach. After the first successful marker-assisted conversion of one commercial maize inbred line to its QPM counterpart for growing in temperate climate [8, 9], this program was continued with this line being used as a donor of the favorable allele in order to obtain adapted QPM hybrids aimed for feed industry. Until now, one hybrid expressed characteristics of a QPM genotype - good grain yield, hard endosperm, high tryptophan and protein contents, good quality index, as well as the stability of tryptophan content in diverse environmental conditions [10]. This hybrid was used in feeding experiments with the objective to test the nutritional and financial effects of replacing

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standard maize with QPM in diets on broiler performances. Improved conversion ratio of feed containing QPM confirmed that, besides being the main energy source, QPM could also be the source of protein and lysine, decreasing the percentage of protein component (e.g. soybean meal) in diets and the need for synthetic lysine supplementation.

Since the discovery of acrylamide in carbohydrate rich cooked foods and its identification as a probable human carcinogen, different mitigation strategies have been taken to reduce dietary acrylamide. However, many of these measures do not maintain the quality and sensory properties of the original product. Attention is therefore turning to improving the raw material by reducing the levels of sugars and/or free asparagine and consequently the risk of acrylamide formation. Genetic approaches to reducing acrylamide risk include the identification of cultivars and other germplasm in which free asparagine and/or sugar levels are low and the manipulation of genes involved in sugar and amino acid metabolism and signaling [11]. The enzymes involved in the biosynthesis and degradation of asparagine are asparagine synthetase, asparaginase and aspartate kinase. The genes that encode these enzymes are all potential candidate genes for the development of genetic markers for breeding programs.

Considering that the high quality is the main goal of today´s crop breeding, the genetic enhancement of nutritional quality in grains is essential to increase the nutritional value and conduct high quality crop breeding [12]. In the future, the focus should be on mainstreaming breeding for nutrient enrichment into breeding efforts to deliver high performing climate-resilient crops with improved nutritional quality to farmers and consumers [13]. In the light of the environmental, health and economic challenges that the world is facing, and given the worldwide setbacks in food security and nutrition, making healthy quality diets is of the utmost importance.

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L IX

Impact of biotic and abiotic stressors on freeasparagine formation in small-grain cereals and maize: Exploring correlations with grain quality and agronomic practices.

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Asparagine functions as a storage compounds in most plants and as key nitrogen transport in the vasculature, likely because it is the amino acid with the highest nitrogen to carbon ratio [1]. Plants acquire inorganic nitrogen from soil (in the forms of nitrate and ammonium) and from the atmosphere (as dinitrogen). Ammonium, is produced through primary nitrate reduction or by absorption from the soil and symbiotic dinitrogen fixation in legume root nodules. Glutamine and glutamate are the primary assimilation products of ammonium. Asparagine synthetase utilizes ATP hydrolysis to synthesize asparagine from aspartate, utilizing either glutamine or ammonium as amino donors. The expression of asparagine synthetase and asparaginase genes are responsive to the environment, being upregulated and downregulated, respectively, by high levels of nitrogen fertilizer [2]. The application of nitrogen fertilizer is essential for achieving high yields, ensuring food security, and attaining the necessary protein levels for breadmaking [3]. However, its use in both conventional and organic farming systems has been associated with elevated levels of free asparagine in the grain [4]. Varietal differences in the response to nitrogen application, regarding protein and asparagine accumulation, underscore the importance of asparagine screening in plant breeding programs to develop reliable recommendations.
Plant stress also triggers a notable increase in asparagine levels, evidenced by the upregulation of asparagine synthetase genes and/or the downregulation of asparaginase genes across various plant species. This accumulation of asparagine during stress primarily occurs through de novo synthesis rather than mere proteolysis or amino acid breakdown. The transcriptional regulation of asparagine synthetase predominantly governs this process, indicating that the level of asparagine accumulation is intricately linked to the expression of its synthetase genes [5]. While the regulation of asparaginases remains less explored compared to synthetases, their activity likely influences asparagine accumulation. Asparaginase activity appears responsive to environmental cues, and diverse stressors such as nutrient deficiencies, salt, water stress, and diseases prompt the accumulation of asparagine across a wide array of plant species [6,7]. The natural senescence or stress induced by pathogen and insect attacks locally leads the plant to break down reserves and translocate them, thereby mobilizing endogenous nitrogen by hydrolysing protein and locally increasing the ammonium concentration. Also, various abiotic stressors can trigger generalized energy stress [8] and proteolysis [9], leading to a reduction in cellular hexose levels and an increase in ammonia. Asparagine accumulation plays a crucial role in nitrogen remobilization and ammonia detoxification during abiotic stress. Beato et al. (2014) [10] propose that asparagine accumulates for these purposes due to a high ratio of ammonia to hexose in the cell. Consequently, the surplus ammonia generated by proteolysis can be recycled into amino acids and asparagine, thus averting the toxic accumulation of ammonia. Most mineral deficiencies, excluding nitrogen and possibly molybdenum, promote an increase in the soluble nitrogen fraction, notably leading to elevated levels of amides, glutamine, and particularly asparagine

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[7]. Accumulation of asparagine is documented in plants deficient in potassium, sulfur (e.g., tomato, alfalfa, mint, rye grass), phosphorus (e.g., oats, mint, rye grass), and magnesium (e.g., tobacco), as well as in deficiencies of most micronutrients [11-12]. Potassium is essential for the functioning of certain asparaginases, with the activity of potassium-dependent asparaginase negatively regulated by glutamine [13,14]. Both potassium-dependent and -independent forms of asparaginase have been identified [15], potentially applicable in wheat. Magnesium is essential for some asparagine synthetase functions. Curtis et al. (2019) [16] identified seven putative asparaginase genes in wheat and demonstrated that one of these responded to sulfur deficiency in the embryo and endosperm. Adequate sulphur application is another important part of a fertiliser regime in order to reduce free asparagine levels in wheat, as increasing application rates are known to reduce asparagine accumulation [17,18]. Similarly to the relationship between nitrogen and asparagine, the effect of sulphur on asparagine levels also varies greatly depending on the wheat genotype [17].

Many water-stressed plants accumulate asparagine (see [7] for literature). Yadav et al. (2019) [19] suggested that asparagine accumulates as a result of drought-induced senescence. Wheat varieties with lower drought tolerance exhibit greater drought-induced senescence, leading to increased levels of asparagine and upregulation of the asparagine synthetase gene expression. In Agrostis stolonifera, asparagine accumulates under saline conditions as reported in [7]. Maaroufi-Dguimi et al. (2011) [20] suggested that asparagine accumulation plays a role in salt tolerance, while Rashmi et al. (2019) [21] suggest that asparagine may function as an osmolyte.

The impact of both biotic and abiotic stressors on free asparagine accumulation in grains constitutes a complex interplay of environmental cues, genetic responses, and agronomic practices. The regulation of asparagine synthetase and asparaginase genes underpins this accumulation process, bearing significant implications for grain quality and crop productivity. Understanding these correlations is essential for optimizing agronomic practices, mitigating stress-induced asparagine accumulation, and ensuring food security amidst evolving environmental conditions.

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L X

Usage of unmanned aerial vehicle (UAVs) in yield and quality prediction and cereal crop monitoring

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Over the past decade, there has been a notable increase in the integration of remote sensing technology in agriculture, particularly in crop production. Unmanned Aerial Vehicles (UAVs) have emerged as valuable tools for scanning fields to detect anomalies in plant health, providing a comprehensive aerial perspective of fields and orchards. Equipped with various types of cameras, UAVs offer a convenient and efficient method for rapidly assessing plant conditions across large land surfaces. Their ability to revisit fields and capture high-resolution images at closer distances to plants compared to other airborne platforms has made UAVs a popular choice among precision agriculture researchers [1,2]. The most commonly utilized optical sensor for this purpose is the multispectral camera, which contains several single-band optical sensors that provide unique values for each band. These bands include those capturing the visible spectrum (red, green, blue) as well as parts of the spectrum invisible to the human eye, such as far and near-infrared bands and the red edge. These bands are crucial for developing new methods to assess plant health and predict yield. Combining bands from both the visible and invisible spectra in various mathematical equations yields vegetation indices (VIs), which are simple yet effective tools for quantitative and qualitative evaluation ofvegetation cover, vigor, growth dynamics, and other applications [3].
One of the most widely used VIs globally over the past 30 years is the Normalized Difference Vegetation Index (NDVI), calculated using red and near-infrared (NIR) bands. NDVI directly correlates with the photosynthetic activity of chlorophyll in plants. Numerous studies on plant phenotyping have examined the linear regression between NDVI and various plant characteristics, such as leaf area index (LAI), biomass, height, and yield, to track seasonal and perennial changes in these parameters. NDVI is particularly useful for characterizing canopy growth or vigor [3]. In addition to NDVI, numerous other mathematical formulas using different bands of electromagnetic spectrum have demonstrated correlations with different abiotic and biotic factors related to plant development and health. Examples include the normalized difference red edge (NDRE), green normalized difference vegetation index (GNDVI), soil-adjusted vegetation index (SAVI), modified soil-adjusted vegetation index (MSAVI), modified chlorophyll absorption in reflectance index (MCARI), enhanced vegetation index (EVI), and simplified canopy chlorophyll content index (SCCCI), among others. When combined with machine learning algorithms, multispectral cameras can be used to determine many different plant characteristics on the field. For agronomists, this is the very useful commercial tool of the future. Many scientific experiments have already been conducted, proving that this future may be nearer than we think.
Crop productivity is frequently constrained by various environmental stresses. Unmanned Aerial Vehicles (UAVs) have emerged as valuable tools for detecting both abiotic and biotic stresses in crops. Early detection of crop diseases is critical for preventing potential losses in both yield quality and quantity. Monitoring for biotic stressors, such as plant diseases, is primarily conducted using three approaches. The first approach involves statistical-based methods utilizing correlation and regression analyses. The second approach utilizes conventional machine learning methods, which rely on traditional supervised or unsupervised techniques and use vegetation indices (VIs) as input features

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to build disease estimation models. The third approach involves deep learning methods, which use raw images along with other features to train the model [2]. These techniques can also be applied to estimate other abiotic and biotic stress factors in fields. Terentev et al. (2023) [4] conducted an experiment on the early detection of wheat leaf rust (*Puccinia triticina*) using a hyperspectral camera in a laboratory environment. They selected different bands from the hyperspectral range. The knowledge gained from experiments like these is a stepping stone for applying the same technique in field conditions, using UAVs to carry the camera.

UAV-assisted thermal and multispectral remote sensing has the potential to detect biophysical characteristics of weed biotypes during the growing season. This includes distinguishing glyphosate-susceptible and glyphosate-resistant weed populations based on canopy temperature and deep learning-driven weed identification algorithms [5]. Multispectral cameras on UAVs enable various predictions and assimilations using machine learning and statistical-based methods. Several studies have found significant correlations between grain yield and VIs, which can be used to estimate important traits such as grain yield under heat and/or drought conditions [6-12]. Airborne estimation of the chemical composition of plants is also possible. Santana et al. (2023) [13] demonstrated how to classify soybean genotypes for primary macronutrients (nitrogen (N), phosphorus (P), and potassium (K)) using a UAV with a multispectral optical sensor and different machine learning techniques, utilizing several VIs. The detection of specific plant chemicals is also possible using UAVs with airborne multispectral sensors. Multispectral measurements of plants in the field successfully predicted the free asparagine content of grain with an accuracy of 71% [14]. While this studie employed proximal sensors, similar results may be achievable using aerial platforms.

In conclusion, the integration of UAV-assisted thermal and multispectral remote sensing has revolutiniozed agriculture over the past decade, offering valuable tools for detecting biophysical characteristics, monitoring crop health, and estimating important traits such as grain yield. These technologies hold great promise for improving crop management practices and addressing various environmental stresses in agriculture.

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L XI

Acrylamide – state of play in Serbia (regulatory compliance with the EU and control of the food on the market)

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Acrylamide is a substance that is produced naturally in foods as a result of high-temperature cooking (e.g. baking, grilling, frying), and has been found in a wide variety of cooked foods, including those prepared industrially, in catering and at home. It is found in staple foods such as bread and potatoes as well as in other everyday products such as crisps, biscuits and coffee.
Following the discovery of acrylamide in food, the industry and other stakeholders, including regulators, took action to investigate how acrylamide is formed and the possible methods that can be employed to reduce levels of acrylamide in foods using the “As Low As Reasonably Achievable” (ALARA) principle.
Acrylamide is formed via the reaction of asparagine and reducing sugars, at temperatures higher than 120°C and the amount of acrylamide formed depends on temperature, baking time and recipe. Its presence in food potentially increases the risk of developing cancer for consumers in all age groups. It is therefore of major importance for the protection of public health that mitigation measures are applied to reduce the levels of acrylamide in food as low as reasonably achievable.
At the end of November 2017, the European Union (EU) adopted Regulation no. 2017/2158 which establishes measures to reduce the risk of acrylamide formation in food by applying certain technological processes and defines reference values as an indicator of the success of the applied measures.
In accordance with Regulation no. 2017/2158, the Republic of Serbia adopted the Rulebook on maximum concentrations of certain contaminants in food ("Official Gazette of the RS", No. 81/19) which, among other things, at the first time in our country prescribed the same measures to reduce the risk of acrylamide formation in foods, identified foods at risk of acrylamide formation, as well as reference values and requirements regarding sampling and testing as in the EU
Also, the Ministry has developed a Guidance for the implementation of the aforementioned rulebook, based on the Guidance on the implementation of Commission regulation (EU) 2017/2158 of 20 november 2017 establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food. This Guidance is intended both for food business operators (FOBs) as a support for the implementation of regulations, and also for the authorities responsible for the implementation of official control at assessment of the FOBs self-control system.
Since 2018 (with the exception of 2020), the Ministry controls the presence of acrylamide in food on the basis of annual food safety monitoring programs of food of plant and mixed origin. Through monitoring programs in 2019, 2021, 2022, and 2023, 279 samples were sampled and tested for the presence of acrylamide, of which 259 samples were compliant and 20 samples were non-compliant (crackers, biscuits, roasted almond snack, waffle sheet and potato chips). After non-compliant samples, official sampling followed and the application of appropriate measures to reduce the presence of acrylamide in food.

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In 208 and 2019, crackers and biscuits were sampled, in 2021 croissants, baked almonds and rice crackers, in 2022 bread, croissants, waffle sheets and snack products, and in 2023 bread, croissants, double cakes, gingerbread, waffle products, waffle sheets and potato chips.

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L XII

Dietary acrylamide exposure in different Serbian population categories

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Acrylamide is a chemical that is naturally formed during the preparation of starchy food products at high temperatures (e.g. frying, baking). Precursors for its formation are sugar and the amino acid asparagine in a chemical process called the Maillard reaction. The amount of acrylamide depends on several factors - concentration of precursors, temperature, heat treatment time and humidity. Acrylamide was first found in food in 2002 [1]. Foods that contain a relatively high content of acrylamide are coffee, potato chips, biscuits, cereals and bakery products [2]. So far, only the neurotoxic effect of acrylamide has been proven in humans, while toxicities related to the reproductive system and genetic material and carcinogenicity have only been proven in animals [3]. The International Agency for Research on Cancer (IARC) classified acrylamide in group 2A, - probably carcinogenic to humans [4]. The fact that acrylamide is found in foods that have a significant share in the human diet caused the need to control its concentration as well as to examine the level of intake through food.

The aim of this paper is to evaluate the acrylamide intake of different population groups in Serbia and to assess the risk of its adverse effects. During 2021 and 2022, 5 food groups from the Serbian market with the highest potential concentrations of acrylamide were examined. 54 samples of bread, 202 samples of potato chips, 58 samples of breakfast cereals, 85 samples of biscuits and related products and 44 samples of coffee were tested. The analysis was carried out using the accredited (ISO 17025) LC MS/MS method on the SHIMADZU LCMS 8040 instrument. The highest average concentration of acrylamide was obtained in potato chips - 664.1 µg/kg, then in biscuits - 99.5 µg/kg, while the lowest concentration was measured in coffee – 1.7 µg/kg. For the purpose of calculating the intake of acrylamide, we combined the obtained results with data from the EFSA Comprehensive European Food Consumption Database on the intake of certain foods for 7 population groups [5]. The obtained values of average intake are shown in table 1.

The highest intake values were observed for toddlers with a share of biscuits, cakes and cookies of 26.4% of the total intake, while the lowest value was determined in elderly (exposure through bread consumption). It is noticeable that younger population shows a higher level of acrylamide intake, which can be explained by dietary habits and the fact that potato chips (food with the highest average acrylamide content),= is consumed in significant quantities at younger ages. The obtained results of acrylamide intake in Serbia is similar to the values obtained in diet studies in Poland, Denmark and Japan, and lower than the intake found in studies in the USA, France and Great Britain [6].

In their 2010 study, Tardiff et al. recommend the acrylamide tolerable daily intake (TDI) of 40 µg/kg bw (in order to avoid neurotoxicity), while in order to avoid the carcinogenic effect, they recommend a maximum daily intake of 2.6 µg/kg bw/day. [7]. Comparing the recommended values with those obtained in our study, we can conclude that there is no increased risk of acrylamide intake.

Since acrylamide is considered a genotoxic and carcinogenic compound, EFSA concludes that a tolerable daily intake (TDI) of acrylamide in food can not be set. Instead of TDI, EFSA recommends applying the Benchmark Dose Lower Confidence Limit ($BMDL_{10}$), which for acrylamide in terms of

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carcinogenicity is 0.17mg/kg bw/day, while for other effects the $BMDL_{10}$ value is 0.43 mg/kg bw/day [8]. By comparing the $BMDL_{10}$ to human dietary exposure , a "level of health concern" known as the margin of exposure (MOE) can be established. For genotoxic and carcinogenic compounds, the following equation is used in order to to calculate the MOE:

MOE = BMDL/Estimated Exposure Dose

EFSA's Scientific Committee states that the MOEs for the cancer-related effects of acrylamide range from 425 for average adult consumers down to 50 for high-consuming toddlers [2].

The results obtained in our work are above the recommended values, and we can conclude that no potential risk for public health is present, with a note that the study did not include all foods containing acrylamide, especially food items prepared in the household using certain thermal treatments, which can significantly contribute to the total intake of acrylamide.

Table 1. Intake of acrylamide, MOE and max share in food intake

Population group	Average intake, µg/kg bw/day	MOE (BMDL – 0,17 mg/kg bw/day)	Max share in intake - %
Toddlers	0.620	274	Biscuits, cakes and cookies – 26.4
Other children	0.479	355	Potato chips – 50.4
Adolescents	0.255	667	Potato chips – 57.8
Adults	0.115	1478	Potato chips – 25.4
Elderly	0.081	2099	Bread – 47.4
Pregnant women	0.121	1405	Potato chips – 25.2
Vegetarians	0.110	1545	Breakfast cereals – 35.3

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[2] Acrylamide in food, available at https://www.efsa.europa.eu/sites/default/files/corporate_publications/files/acrylamide150604.pdf

[3] J. H. Exon, A Review of the Toxicology of Acrylamide, Journal of Toxicology and Environmental Health, 9 (2006) 397-412.

[4] IARC List of Classifications, available at https://monographs.iarc.who.int/list-of-classifications/

[5] The EFSA Comprehensive European Food Consumption, available at Databasehttps://www.efsa.europa.eu/en/microstrategy/foodex2-level-3

[6] C.A.G. Timmermann, S.S. Mølck, M. Kadawathagedara, A.A. Bjerregaard, M. Törnqvist, A.L. Brantsæter, M. A Pedersen, Toxics, 9 (2021) 155.

[7] R. G. Tardiff , M. L. Gargas, C. R. Kirman, M. L. Carson, L. M. Sweeney, Food and Chemical Toxicology, 48 (2010) 658.

[8] EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2015. Scientific Opinion on acrylamide in food, EFSA Journal, 13(6):4104 (2015) 321.

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PRACTICAL SESSIONS

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PS I

Application of molecular biology techniques for identification and expression profiling of asparagine synthetase genes in cereals

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1. Molecular biology technique for detecting presence/absence of asparagine synthetase genes in cereals – qualitative PCR (Polymerase Chain Reaction)

DNA extraction

Genomic DNA extraction commercial kit **NucleoSpin Plant II, Mini kit for DNA from plants, Macherey-Nagel, Sweden:**

General steps of DNA extraction from plant tissue (leaves, embryos, developing seeds) using commercial kits:

Step 1: Cell Lysis

Step 2: Purification – Binding Nucleic Acids to the Column

Step 3: Washing

Step 4: Dry Spin for Ethanol-free DNA

Step 5: Elution

Figure 1. Steps in DNA extraction

Figure 2. Assesing DNA integrity and purity

Following steps:

Dissolving extracted DNA in TE buffer (10 mM Tris·Cl, pH 8.0. 1 mM EDTA)

Testing intergrity:
resolving DNA extracts on 0.8% agarose gel

Testing amount and purity:
measuring DNA concetration on BioSpectrometer, Eppendorf
checking A260/A280 and A260/A230 ratio

Setting up PCR reaction:

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Adding required reagents and template (genomic DNA) to PCR tubes in optimal concentrations for efficient PCR amplification of DNA templates

Components of PCR master mix:

- reaction buffer
- $MgCl_2$
- primers
- Taq Polymerase
- dNTPs
- template DNA
- nuclease free water

Mixing and brief centrifugation of master mix

Thermal cycling for PCR:

Amplifying template DNA per thermocycler and primer parameters

Setting the PCR machine parameters:

- Number of required thermal cycles for efficient amplification
- Temperature for the following steps:

- denaturation
- primer annealing
- extension

Starting PCR machine (Thermocycler)

Figure 3. Thermocycler

Evaluating amplified DNA by agarose gel electrophoresis

- preparing agarose gel of requred percentage
- loading molecular weight ladder into the first lane of the gel
- loading samples
- running electrophoresis
- staining gel in EtBr solution
- gel imaging under UV light

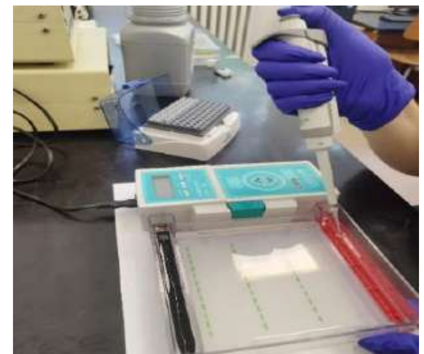

Figure 4. Agarose gel electrophoresis

Data analysis

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- **Estimating aproximate size of the resulting bands – comparing the migration dsitance of hte band to the migration distance of the fragments in the ladder**
- **Scoring bands for presence/absence**

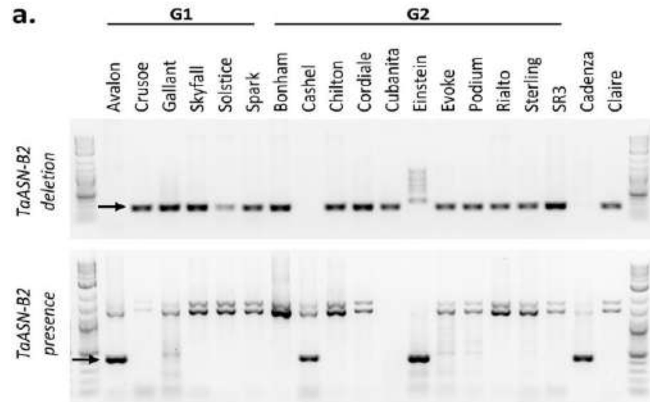

Figure 5. An example of agarose electropohoresis results - Presence/absence of *TaASN-B2* in UK wheat varieties. a. UK Flour Millers milling group 1 and 2 hard bread. The distinguishing PCR products are indicated with arrows.*

***Reference:** J. Oddy, R. Alarcón-Reverte, M. Wilkinson, K. Ravet, S. Raffan, A. Minter, A. Mead, J. S. Elmore, I. M., de Almeida, N. C. Cryer, N. G. Halford, S. Pearce, BMC Plant Biology, 21 (2021) 302.

2. Molecular biology technique for analyzing expression level of asparagine synthetase genes in cereals – quantitative RT PCR

RNA extraction and purifying

Total RNA extraction commercial kit **GeneJET Plant RNA Purification Kit, ThermoFisher Scientific**

General steps of RNA extraction from plant tissue (leaves, embryos, developing seeds) using commercial kits:

Step 1: Cell Lysis

Step 2: Purification – Binding Nucleic Acids to the Column

Step 3: Washing

Step 4: Dry Spin for Ethanol-free RNA

Step 5: Elution

Following steps:

RNA purification using commercial **kit**
Ambion® DNA-free™ DNase Treatment and Removal Reagents

Testing intergrity:

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resolving RNA extracts on 1% agarose gel

Testing amount and purity:
measuring RNA concetration on BioSpectrometer, Eppendorf
checking A260/A280 and A260/A230 ratio

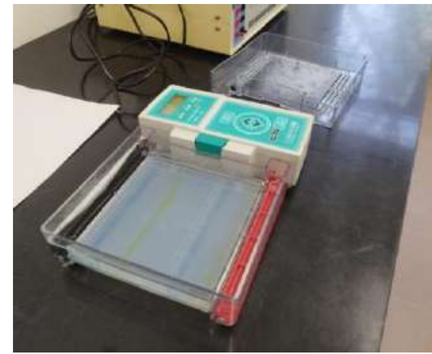

Figure 6. Agarose gel electrophoresis - RNA

cDNA synthesis

cDNA synthesis using commercial kit **Ambion® DNA-free™ DNase Treatment and Removal Reagents** and **RevertAid Reverse Transcriptase (200 U/µL), ThermoFisher Scientific**

Components of reaction mix:

- RevertAid Reverse Transcriptase, 200 U/µL
- reaction Buffer
- random hexamer primers
- RiboLock RNase Inhibitor, 20 U
- dNTP mix
- template RNA
- nuclease free water

Verification of cDNA on agarose gel

quantitative RT PCR

qPCR reaction set up

- components of qPCR reaction
- commercial master mix 5x HOT FIREPol® EvaGreen® Mix Plus, Solis BioDyne
- primers
- template – cDNA
- nuclease free water

Figure 7. Setting up qPCR reaction

Setting up the qPCR reaction on qPCR machine

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- setting up the experiment
- plate set up
- setting up run conditions
- setting up the reaction
- starting the procedure

Figure 8. qPCR machine

Data analysis

- interpretation of amplification plot of qPCR
- calculation of relative gene expression

2^[-delta delta C(T)] method

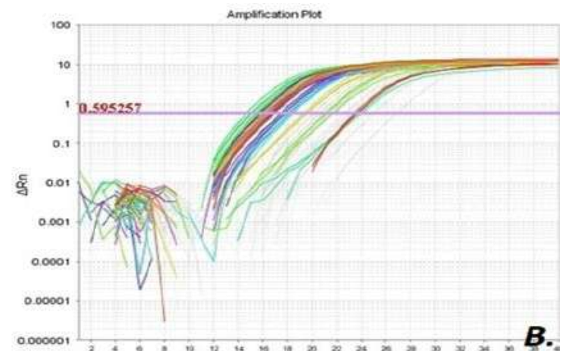

Figure 9. Graphical presentation of qPCR results

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PS II

Amino acids analysis using HPLC in cereal products

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Free (soluble, nonprotein) asparagine can be converted into the processing contaminant, acrylamide, during high-temperature cooking and processing, and this has led to ongoing efforts to reduce free asparagine concentration [1]. According to the literature, asparagine content in cereals is influenced by many factors such as genetic background, growing conditions, harvest time and post-harvest storage conditions [2]. Therefore, accurate measurement and monitoring of asparagine levels in cereal products are essential to develop effective strategies to reduce acrylamide formation and improve food quality.

Instrument: Thermo Scientific UltiMate 3000 HPLC system

Chromatographic conditions:
Column: *Hypersil GOLD Amino C18 column (250 mm × 4.6 mm, 5 µm), Thermo Scientific, USA*
Mobile phase: *solvent A: 40 mM Na2HPO4, pH 7.8 and solvent B (acetonitrile/metanole/water, 46:46:10, v/v/v)*
Gradient: *0% B 0-1.9 min, 57% B 1,9-18.1 min, 100% B 18,1-22.3 min, 0% B 22.3-26 min.*
Flow: *2 mL/min*
Column temperature: *40°C*
Injection volume: *10 µL*
Detection: *UV-Vis (UVD)****ASN calibration curve:***

A standard curve of asparagine (ASN) was prepared in the range of 10-60ppm.
to 10mL volumetric flask add:

+1200, 1000, 800, 600, 400, 200µL solution of aminoacids, respectively (100ppm)
+ fill in with 800, 1000, 1200, 1400, 1600, 1800 µL of water, respectively

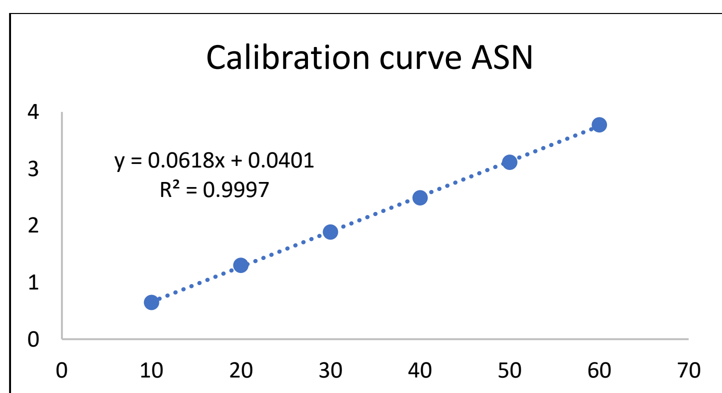

Fig. 1 Calibration curve for calculation of asparagine

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Sample preparation:

1. 1.0000g finely ground dry sample is weighed in 15mL centrifuge tube and mixed with 5mL of water. The mixture is shaked for one hour used shaker (Multi Reax Vibrating Shaker) at 2000 rpm for 60 minutes at room temperature and than centrifugated at 10 000rpm for 5 minutes at 4°C.

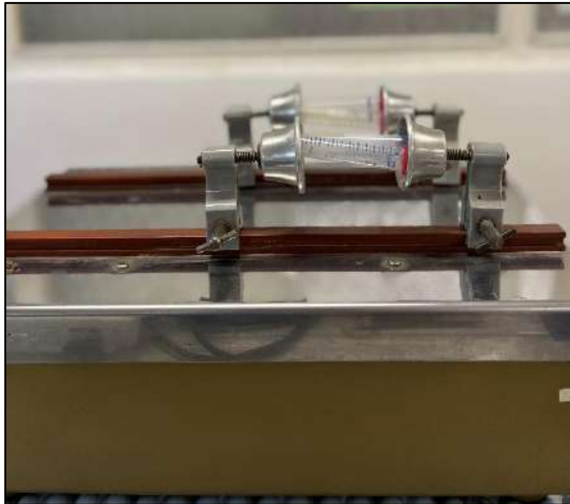
Fig. 2 Shaking sample with water

Fig. 3 Centrifugation of samples

2. After centrifugation 1 mL of supernatant is transferred into a eppendorf tube contening 500µL Acetonitrile and vortexed. Mixed sample leave in the refrigerator for 2 hours. After cooling the mixture is centrifugated at 10 000rpm for 5 minutes at 4°C.

Fig. 4 Transfer the supernatant to eppendorf tubes

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3. 500µL clear supernatant is tranfered into a glass tube containg 500µL o-phthaldialdehyde solution (OPA) for derivation. Derivatization was performed before the very beginning of the analysis by vortexing for 1 min. The *o*-phthaldialdehyde solution (OPA) was prepared by OPA reagent, MeOH, Borate Buffer (40mM, adjust to pH 8.5 with NaOH solution (10 N)) and 2-Mercaptoethanol (2-MPA).

Fig. 5 Transferring the supernatant to a glass tube

4. After filtration through a 0.45µm pore size nylon syringe filter were samples injected and analyzed by HPLC- VID

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Fig. 6 Putting samples into HPLC according to the given sequence

Chromatogram results:

Asparagine analysis was performed by calculating the peak area by linear regression from an external six-point standard curve.

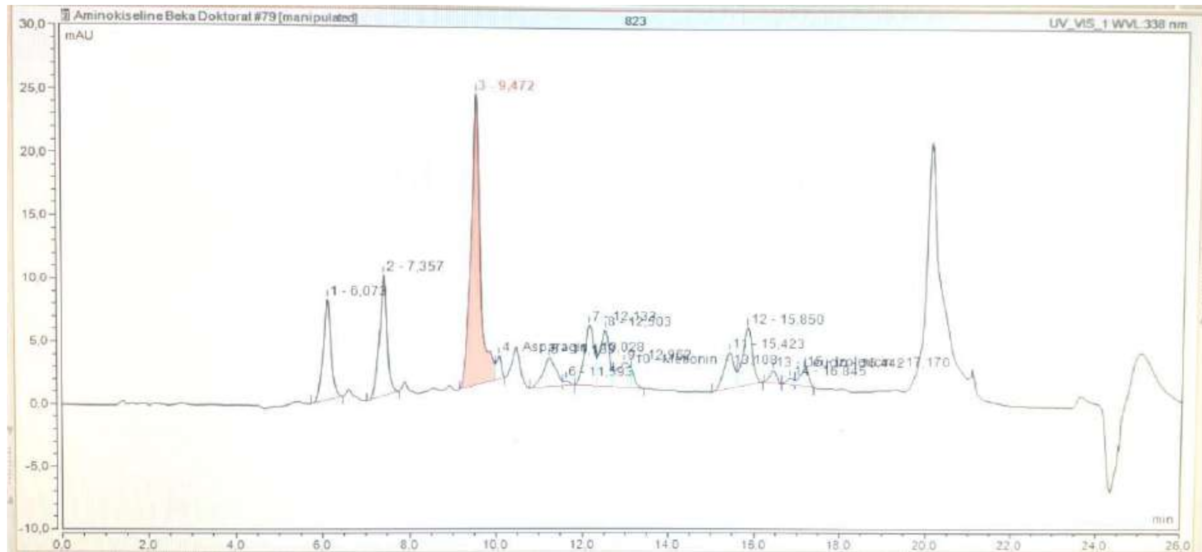

Fig. 6 Chromatogram of wheat sample, marked asparagine peak

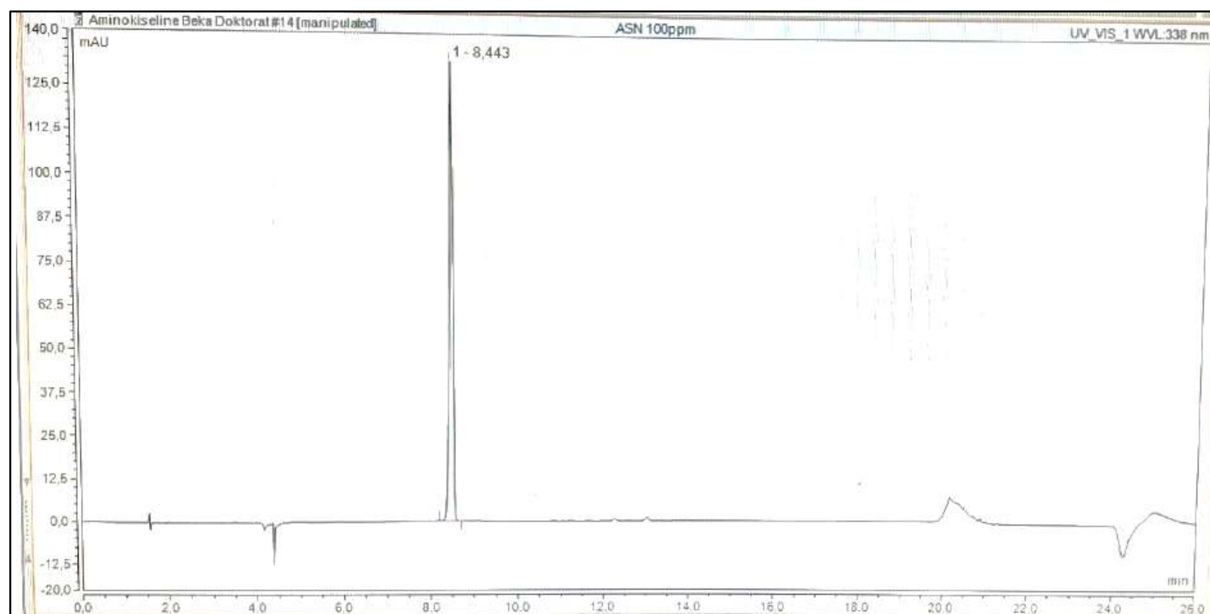

Fig. 7 Chromatogram of asparagine (ASN) standard

References:

[1] J. Oddy, M. Chhetry, R. Awal, J. Addy, M. Wilkinson, D. Smith, N.G. Halford, Genetic control of grain amino acid composition in a UK soft wheat mapping population. The Plant Genome (2023), 16(4).

[2] S. Žilić, I.G. Aktağ, D. Dodig, M. Filipović, V. Gökmen,. Acrylamide formation in biscuits made of different wholegrain flours depending on their free asparagine content and baking conditions. Food research international, (2020) 132.

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PS III

Color analysis of cereal-based food products by Chroma meter Konica Minolta

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The color of a food item is a valuable parameter in food quality control given the fact that it may indicate the presence of certain compounds, such as products of the Maillard reaction that occur during thermal treatment along with nonenzymatic browning, i.e., acrylamide or hydroxymethylfurfural (HMF), etc. Thermal processing conditions also have an impact on the color of the products [1].
Numerous studies have indicated that the chromatic parameter a* can be a useful predictor of acrylamide formation. Mesias et al. [2] used the color parameter a* to discriminate French fries according to their acrylamide contents as 'below' or 'above' benchmark level set at 500 µg/kg for fried potatoes by EU regulation. A value of 0.855 for a* was found as the threshold value for acrylamide content above the benchmark level. Analyzing the free amino acid in cereals, Kocadağlı et al.[3] found that the free Asn content was lower in different colored corn grains than in wheat and hulless barley. Žilić et al.[4] reported a high variation in the free Asn content between the examined corn genotypes, from 190 mg/kg in red kernel corn to 471 mg/kg in yellow kernel corn. Additionally, certain natural compounds present in corn, depending on the genotype, can affect the acrylamide formation in corn-based thermally treated foods. The study carried out by Delgado et al.[5] suggested that corn genotypes rich in anthocyanins and with lower levels of fat and phenolic compounds could reduce the acrylamide formation in tortilla chips. According to the results of Žilić et al.[4] lower content of acrylamide was determined in biscuits prepared from anthocyanin-rich wholegrain flour of red- and blue-colored corn and baked at 180 °C for 7, 10, and 13 min than in white corn- and yellow corn-based biscuits.

The color of cereal flour and baked products can easily be measured and analyzed by Chroma meter Konica Minolta CR-400 and SpectraMagic NX software [6]. The Konica Minolta CR-400 is a tristimulus colorimeter that uses filters to match the spectral response of cones in the human eye and data for standard daylight illuminant D65 to provide a numerical value for the color being measured. It uses filters that match the standard observer color perception and provides numerical and traceable data for color communication and control.

Instrument:
Chroma meter Konica Minolta CR-400, software: SpectraMagic NX

https://www.youtube.com/watch?v=cuRYR8dGkmo&t=42s

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Sample preparation:
Cereal flour was obtained on a laboratory mill for fine sample preparation (Perten 120 L, Perten Hägersten, Sweden) by grinding the kernels to fine powder (particle size ≤500µm).
The flour was then transferred to a measuring vessel and tightly packed for color analysis.

Instrument color measurement:
Color properties of cereal flours were measured with a Chroma meter (CR-400, Konica, Minolta, Japan), calibrated against the white calibration cap CM-A70 that serves as the white calibration standard. Flour samples were transferred into a glass cell made of optical glass for measurements. The color of samples was given as average CIE L*, a*, and b* values of four measurements per sample. CIE L*, a* b* values were defined using the Spectra Magic NX software. In the L*a*b* color space, L* indicates lightness (from black (0) to white (100)). The labels a* and b* represent chromaticity coordinates (color directions), where +a* is the red axis, - a* is the green axis, +b* is the yellow axis, and -b* is the blue axis [7].

Fig. 1. Chroma meter Konica Minolta CR-400

Connecting the chroma meter with the software

- We connect the device to the computer and turn it on
- We put the USB (it is the basic key to recognize the device and the software without which it cannot work)
- Let's open the SpectraMagic NX program
- Turn on the device and at the same time enter the device menu that appears on the display (at the same time, pull the power button and hold Enter on the device, and then press Enter again)
- On the display, use the down arrow to go to PC mode and press Enter (this enables connecting the device with the software)
- In the program, press the icon with a blue circle and the "lightning" sign Connect. When the connection is made, that icon will be red with an **x**, and **y** at the bottom. When the connection is made, that icon will be red, the toolbar will have a blue icon.

Setting up the device and software for operation

- Go to Data, then Observer and Illuminant, select 2 degrees in the field and Primary C.

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(There are more illuminants, we will work with C (Lab). The device is factory set to illuminant C, so it should also be adjusted in program C in the way described above. If we want to change C to e.g. D65, some also use this illuminant, in Primery we will select D65 and when the device is connected to the computer the software will change the illuminant on the device as well)
- The screen template is selected, i.e. the desired way of displaying the results on the screen. There are: Detail, Simple, Standard, and For CR for our work. We will mostly work with Simple. Click on File, then Template, Load Template, select one of the 4 listed and click OK.

Calibration

- First, the white calibration of the device is performed on the WHITE CERAMIC PLATE. When the device is placed on the whiteboard, go to Instrument, then Calibration.
Note: Calibration is performed at the beginning of the measurement, and if the device is turned off for a long time during the day. The white ceramic plate must be stored well and closed and stored after each calibration. When installing, the x, y, z numbers on the white ceramic plate must be entered into the device. In general, this is never done again, only if there is some dangerous error that the device shows. This can be done directly on the device or program, via Instrument - Set Call Data - enter the values and go to the Lab.

Target measurement/opening and sample measurement

- The device is now ready for measurement. First, we measure the so-called Target, a kind of standard that exists primarily because of the industry so that the colors do not deviate from the target. In our case, Target is a sample. However, we have to open the measurement with Target because it determines and names the file in which the samples come. If, during the measurement of the sample, the measurement is interrupted with the target measurement command, a new file will be opened in which the samples to be measured will be entered. All samples within one file are compared to the initial target. Δ of the sample is obtained only concerning the target, and numerical values of the difference between the two samples cannot be obtained. To achieve this, we need to create new files. Target is measured by placing the device on the sample, then clicking Measure Target (icon under the main menu) or Instrument - Measure Target. Then a field appears in which we enter the name of the target, i.e. the name of the file into which further sample measurements are entered. We can type some comment or description of the sample, ie. target. Press **OK**.
- Then we measure the samples. Place the device on the sample, then click Measure Sample (extracted icon under the main menu) OR Instrument - Measure Sample. We get a field in which we type the name of the sample. **OK**
- Note: An optical glass vessel measures powdered, granular, and floury samples. It is important that there is at least half of the sample in the glass and that it is well packed in the lower part so that the light is not scattered. The glass should be washed carefully so as not to cause any damage to the glass. This also applies to the liquid sample cup. Both glasses have their holders. The powder sample cup is a small black attachment that is placed on the device and the cup on top of it. For a liquid sample cup, it is a large support where the device is placed in a horizontal position.
- Through the software, we can set the device to perform several consecutive measurements and output only the average. It is very useful when measuring inhomogeneous and granular samples. Also, between those few consecutive times, we have time to fill the glass with the sample a little more and shake it.
- Target Averaged Measurement and Sample Averaged Measurement (icons dropped below the main menu) OR Instrument – Averaged Measurement -Target Averaged Measurement and Sample Averaged Measurement

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- There is a possibility to give names and numbers of targets and samples before recording. This means that we don't have to name each sample separately if we don't want to. In that option, we give one name, and then all samples have the same name, only a different number. Data - Supplementary Data Information - Auto Naming (target, sample)
- When we press Edit Mode from Tools, or the Edit icon with a triangle that we have drawn, a mass of icons will appear in the lower toolbar that gives us many options for changing the type of graph, color, and font.
- Right-click on e.g. Graph - Property - options for fixing and changing the graph. If there is a small difference in the color of the samples on the graph, the color of the graph can be not given as a spectrum, but the color of that sample prevails. This reduced the range of coordinates. That's why we go to Property L and set it from 0 to 100, and set ab to, for example, 50. The X and Y axes will go from -50 to 50. In this way, we will get an expanded color field on the graph, that is, almost the entire spectrum. Important!!!
- Continuation of measurement after a certain time in the same file, i.e. target. Open the header file and next to the table, there is a window. In that window are the names of all the targets/files that were created. Open the target/file we want, that is, in which we want to insert new measurements and then just measure the sample. So we don't start measuring with a target. All these new measurements can be seen on the graph.
- Data transfer for targets/samples and samples. If from one file/target that has, for example, 10 measurements, we want to compare e.g. target/sample with 1. We need to open a new target/file. We select target - Move to Target (the icon thrown out from under the main menu OR Tool - Move to Target. A new Target/file opens which we name or has the same name only II and it appears in the window next to the table. Now let's return to the old target/file, we select sample 1 and click Change Target. Then the data goes to the new target/file. Now we have a new target/file that contains only target/sample and sample 1 from the old file. So if we want the graph to show us certain samples, and we have a lot of measurements, we can't just select those samples and get a graph, as can be done in Excel, but we have to create a new target/file in the way described above.
- If we want to get some more data in the table of results that we need, and which software enables it, we go to Data - List Item - from the left window, and drag the parameter we need to the right.
- View – Toolbar Setup – Command – when any command is opened, by dragging the icon we need to work with, we place it in a position on the toolbar.

Transfer data to another program
- Data from the table can be copied and transferred to Excel, for example.
- Graphs, that is, the entire screen is copied via the Print Scrn button on the upper part of the keyboard. Nothing can be done here, so the graphs are arranged in the SpectraMagic program and transferred.

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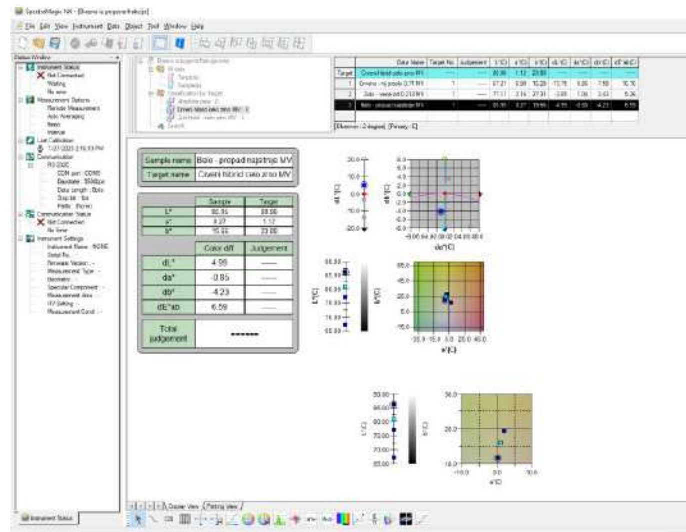

Fig. 2. Color measurement of corn flour samples

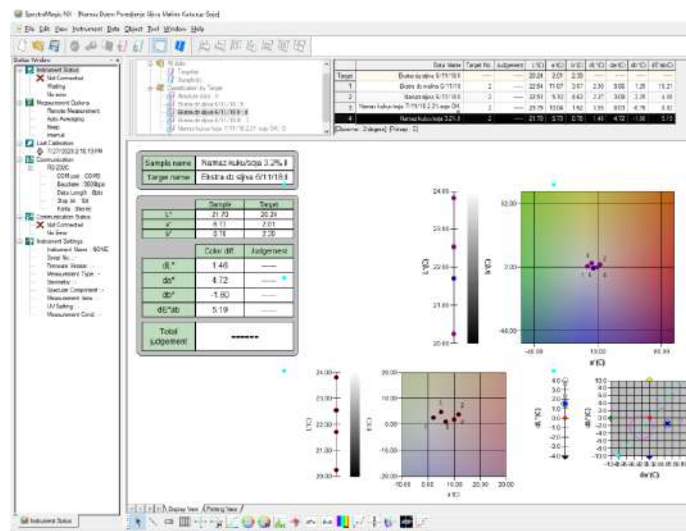

Fig. 3. Color measurement of sweet spreads

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PS IV

Hybridization in the small grains breeding

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One of the basic tasks at the Zemun Polje Maize Research Institute (MRIZP) is the creation of small grain varieties.

Varieties of small grains created in MRIZP are characterized by high potential for grain yield, excellent grain quality, primarily for the production of flour and bread as well as other products used for human and animal nutrition. Other positive agronomic properties also characterize MRIZP small grain varieties: they have increased tolerance to both abiotic (low temperatures, lack of moisture, i.e. drought, lodging) and biotic factors (the most important pathogens and pests in grain). They were created in the agroecological conditions of Serbia and have an advantage over the varieties that were created in some other locations, primarily by adaptability and stability.

Since the beginning of the modern small grain breeding program in 2006 until today, more than 25 varieties have been created. In 2023, 6 MRIZP small grain varieties were released by the Variety Commission of the Serbian Ministry of Agriculture, Forestry, and Water Management.

The breeding program includes the creation of winter bread wheat, where winter wheat is predominantly used in the confectionery industry, and hard wheat (*T. durum*) for pasta production. Spelt wheat (*T. spelta* L.), one of the oldest wheat subspecies used in human nutrition, besides superior nutritional properties, a pleasant taste, and aroma, has become increasingly popular due to high digestibility and suitability for consumers with wheat sensitivity. The barley breeding program includes the creation of varieties of two-row and multi-row barley for the brewing industry and feed production, as well as bare-grain (var. *nuda*) barley for human consumption. The triticale breeding program is based on the creation of varieties for high-quality feed production, but it can also be used in small quantities as an ingredient in flour mixtures with wheat flour for confectionery products. Most of the small grain cultivars created in MRIZP are sown in winter, but two of them Apolon (barley) and Admiral (triticale) could be sown in late autumn or early spring (facultative/alternative).

At the beginning of the breeding process, it is necessary to define the model or ideotype of the variety to be created. The ideotype of the variety is different for different plant species, but it must be harmonized with the MRIZP breeding program and the breeding directions in Serbia and the world. When creating the ideotype of the variety, the following are taken into account: the properties of the variety resulting from its relationship with the external environment (abiotic and biotic factors), the relationship of the variety with other plants (composition), higher efficiency of photosynthesis, economical use of mineral nutrients, higher yield and better quality (via yield and quality components) as well as market requirements.

In order to ensure success in breeding, it is necessary to have a collection (gene pool) that will serve the breeder for crosses. Collections can be permanent and working, the latter being used for the hybridization process.

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The crossbreeding process continues with the selection of parental pairs, that is, with the creation of a hybridization plan. The selection of parent pairs is made on the basis of the selected concept (variety, trait and gene concept). In MRIZP, the concept of trait is used, with a tendency to move to the concept of gene. The concept of properties means the selection of parental pairs based on the knowledge of the properties that are to be combined in future varieties [1].

Hybridization represent crossing of two plants or lines of dissimilar genotype. In hybridization one of the plants is taken as the female plant and other plant as male.

The most widely applied method of crossing in MRI is the method of simple crossing (A x B), where variety A is the female, while variety B is the male of the new line, the variety.

The crossing procedure itself is performed in 2 stages: 1) emasculation of the female flowers and 2) pollination of the female flowers.

Emasculation of the female flowers

The small grains on which the breeding process is carried out belong to monoecious, self-fertilizing plant species with bisexual flowers, which means that the procedure of emasculation of the female flowers represents manual (hand) removal of anthers or entire stamens from flowers (there are 3 in the flower) without damaging the female reproductive organs [2] is carried out by removing the male sexual organs, the stamens of which there are 3 in the flower. The emasculation must be performed on time at the very beginning of flowering when the anthers are green before self-fertilization occurs. After emasculation, the spike must be isolated by placing a pergament paper bag with a mandatory label indicating the name of the parent and the date of emasculation. The parchment paper bag should be closed and secured with a paper clip. Emasculation is performed with scissors and tweezers, and after emasculation, the equipment and hands are disinfected with 98% alcohol.

Pollination of the female flowers

Pollination is carried out when the stamens of the male are ripe (yellow), and most often it is in 3-7 days period, depending on the agrometeorological conditions, the variety, etc. Pollination is carried out with the same equipment as emasculation, by applying the ripe pollen of the male to the unfertilized stigma female's beating. The previously removed insulator bag is returned with the date of pollination and fastened again with a paper clip. Hands and tools are disinfected again with 98% alcohol. The record of the completed crossing is kept in temporary and permanent documents. Crossing can be carried out in the field and the greenhouse, in which case it is necessary to provide a nutrient solution (5% sucrose) and a fungicide 0.5%.

Usually, 3 classes/combinations of crosses are done in one season, while the number of combinations is different and depends primarily on the structure of sexual organs, the concept of choosing parental pairs, the combination abilities of parents, the number of people for crosses (Borojević, 1992). Most often it is 100 + combinations/ plant species.

In this way, F1 grains are obtained that are small, narrow, and shriveled, from which, in the generations of separation of properties from the F2 generation onwards, variability will be obtained from which, by applying the Pedigree selection method, it will be possible to select offspring with the desired recombination of the genes of the parental pairs that have been selected for intersections.

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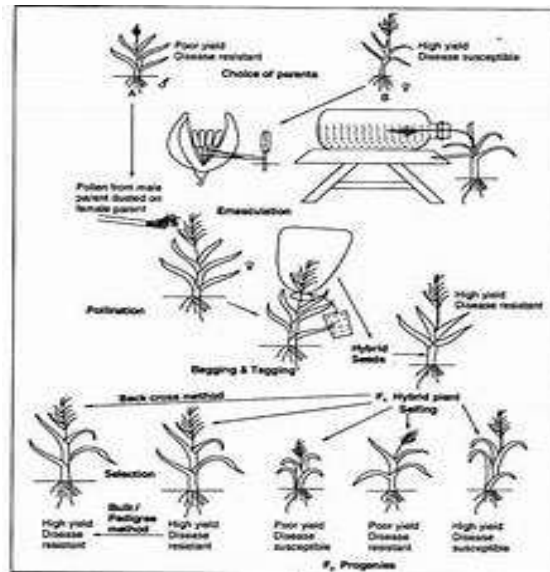

Fig.1. Hybridization process

Techniques of hybridization

Choice of parents: The choice of parents depends on the aim of breeding (improvement of yield, quality, pest and disease resistance, and resistance to abiotic stress).

Evaluation of parents: The evaluation of parents is the necessary conduct for the traits that we need transfer and combine in a new genotype (offspring).

Sowing plan of parent plants: It is necessary to do the sowing of the parents based on the cross-combination plan. If the duration of flowering is the same, simultaneous sowing of both parents can be done. Bearing in mind that in the crossbreeding plan, combinations of genotypes with different flowering times are sown, it is necessary to sow the collection of parents in at least two-timed sowings with a difference of 10 to 14 days.

Emasculation is the procedure for removing of male reproductive organs (stamens or anthers) or removing or killing of pollen grains without damaging the female part of the plant is called emasculation.

Mechanical methods based on flower emasculation techniques (removal of anthers/stamens), followed by pollination with a male parent.

-**Hand emasculation** (in wheat, barley, triticale, oat, sorghum rice)/ manual removal of anthers or entire stamens from flowers without damaging the female reproductive organs [2]. Emaculation should be done before the anthers are mature and before the stigma become receptive. The limits anther dehiscence by physical removal, while deficiency and limits are high possibility of pistil damage and need skills and is labor-intensive [3].

- **Hot-water methods** - represents the treatment of the complete floret with hot-water treatment to render the pollen non-viable. This tecniques based on pollen inactivation with treatmnet by temperature (between 45°C and 50°C for 2 min to 10 min has been used to emasculate) which is slightly lower than that required to inactivate the ovary. The advantage is rapid and technically simple, no mechanical injuries on flower, produces normal seed set, while limitation is possibility to damage egg cells, optimum temperature × time varies with species and even variety [4].

- Cold treatment: Like hot water, the cold water treatement also kills pollen grains without damaging female reproductive organs (gynoecium).

- **Anther aspiration techniques** based on physical removal of anthers from the flower (small size) via a vacuum-suction system. The method requires considerable operating skill to avoid mechanical

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damage to the stigma, which can occur if the suction tip comes into contact with the stigma or style [5](Cordeiro 2008). The advantage: high seed set, but high percentage of self-pollination, while limitations is that is efficient only on a small scale [6].

- **Plastic-bag method** - the florets are covered with a clear plastic bag, which traps heat and increases humidity, causing the temperature to reach lethal levels and prevent the dehiscence of anthers. The which advantage is Cheap and easy, while limitation is moderate levels of self-pollination [7]. The plastic-bag emasculation technique can be successfully adopted in self-pollinating crops, provided that the emasculation and crossing work is undertaken during a suitable season and that the anthesis coincides with the maximum temperatures required for pollen abortion

- **Chemical emasculation (alcohol emasculation and GA3)** are some of the mechanical techniques used to destroy/remove anthers in self-pollinating crops. The 70% ethyl alcohol for 2 min to 5 min to induce male sterility (rice). However, the effective concentration, application period and immersion time are crop species-specific and thus there is a need to optimize emasculation protocol for the species being considered in the breeding program.

Isolation of flower with bag (bagging): After emasculation the flowers or the inflorescence need preserve in suitable bags of appropriate size. Also, after cross pollination is necessary isolate flower with bags of appropriate size to prevent cross pollination. The bags should be made up of impregnated paper or fine cloth.

Labeling (tagging): After emasculation, for each flower need labeling with data od name of genotype, data of emasculation and person who made emasculation. The label (tag) should be appropriate size and shapes (3 x 2 cm or 3 x 3 cm) and may be from different material (paper, plastic).

Pollination is conducted by applying the anther which dehiscence and releases pollen grains placed on the female reproductive organ this process is called pollination. The pollination together with emasculation determine efficiency of hybridization and obtained number of viable seeds.

Harvesting and storing of F1 seeds: The crossed ears need harvest at the maturity stage of genotypes and obtained seed need harvested and seeds were dried and stored for further use. The seeds from each cross should be stored separately to protect from mixing.

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PS V

Drone imaging of sulfur treated field

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UAV (Unmanned Aerial Vehicle) equipped with multispectral camera is used to detect difference in cereal crops with different sulfur treatments.
Sulfur is an essential nutrient for plant growth, and its deficiency or excess can impact crop yield and quality. By applying different sulfur treatments to cereal crops and using UAVs equipped with multispectral cameras, researchers can analyze how these treatments affect crop health and vigor. Differences in reflectance patterns between treated and untreated areas can indicate variations in crop condition, such as differences in chlorophyll content or stress levels.

Equipment:

- **DJI P4 Multispectral drone**: a high-precision drone with a seamlessly integrated multispectral imaging system built for agriculture missions, environmental monitoring, and more.
- **D-RTK 2**: High Precision GNSS Mobile Station is a durable GNSS receiver that supports all major global satellite navigation systems
- **"DJI GS Pro"** mobile application on Apple Ipad device

1. Programing the Flight Mission in "DJI GS Pro" mobile app.

- - Draw the polygon of the experimental field in the DJI GS Pro mobile application and name the mission.
- - Adjust flight altitude. Optimal altitude depends on the size of the experimental field, height of the objects overflown (plants), type of the terrain, and requirement for the specific spatial resolutions. For this particular experimental field, a low altitude of 15-20m is optimal for image analysis (Fig. 1).

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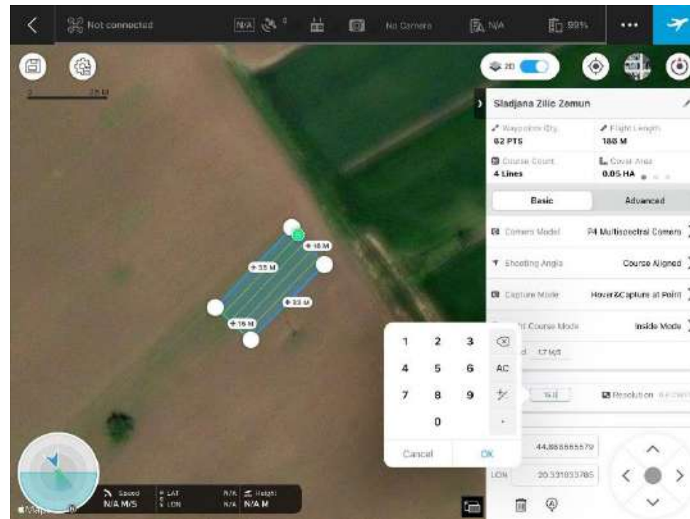

Fig. 1. Entering the altitude of the flight to DJI GS Pro app

- Adjust both the Front and Side Overlap among consecutively captured images to a fixed value (75-80%), and Course Angle (Fig.2).

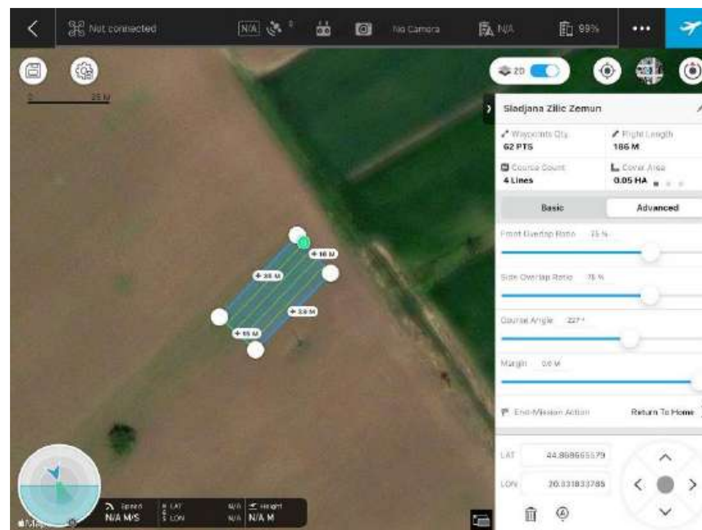

Fig. 2. Adjustment of the front and side overlaps, course angle and Margin

2. <u>Flight preparation</u>

- Turn DJI P4 Multispectral drone on
- Turn D-RTK 2 on and connect the RTK with the drone.
- Calibrate the drone compass (Fig.3)

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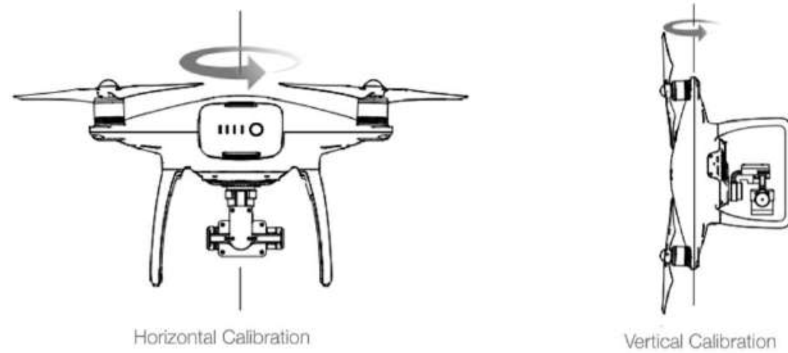

FIg.3. Compass calibration

3. <u>Flying</u>

4. <u>Image stitching – creation of the ortomosaic</u>

- Transfer images from the microSD card from the DJI P4 Multispectral drone to the computer.
- Put TIF and JPG files in different folders.
- Visit the website: https://solvi.ag and log in with your email address.
- Create new project (+Farm)
- Upload and overlap the images on the Solvi web application (Fig. 4-6)
- Receive email with processed data (Fig. 7)

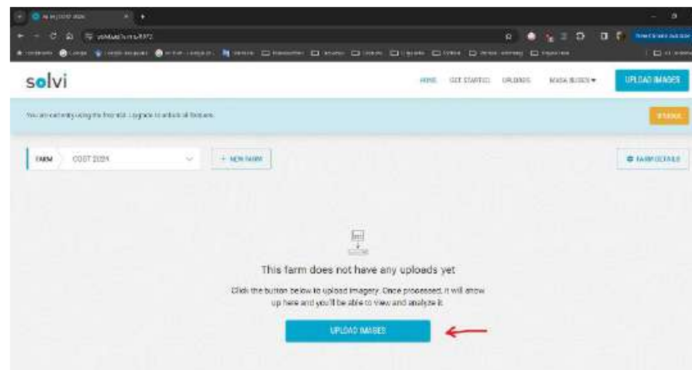

Fig. 4. Uploading images on Solvi web app

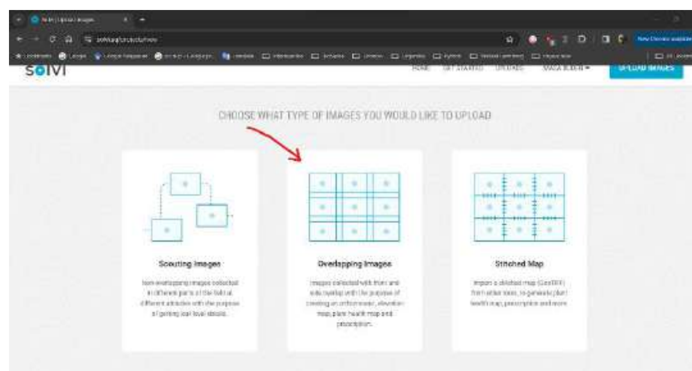

Fig. 5. Overlap the images

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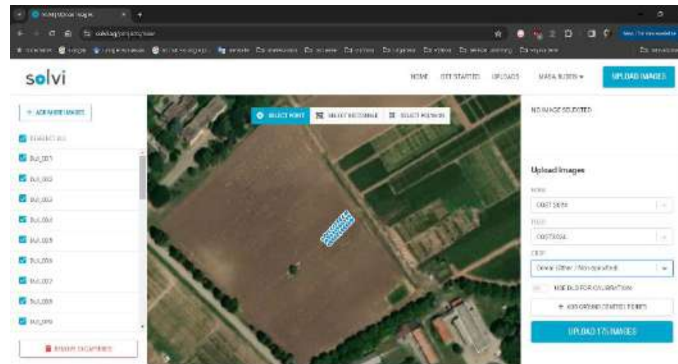

Fig. 6 Enter the name of the field and crop type

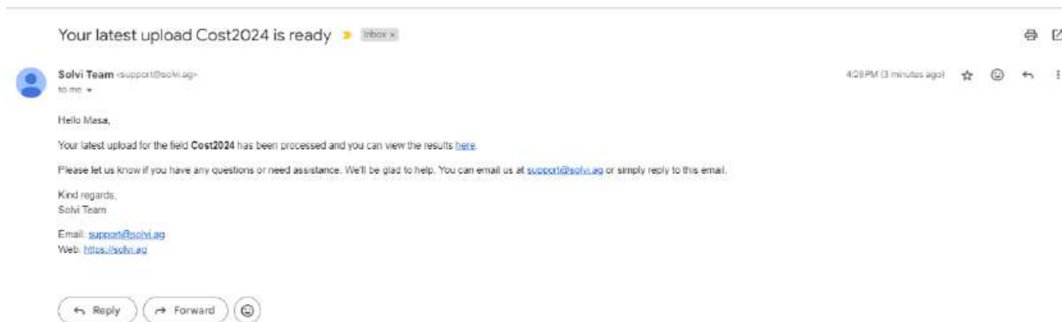

Fig. 7. Email with processed data

5. Data analysis

Calculating various Vegetation Indices (VIs) can be achieved through numerous methods. Numerous GIS software packages, for example QGIS, ArcGIS, etc. Enable the calculation of Vis for each pixel captured by a multispectral camera. Each pixel contains numerical values representing the amount of reflected light for each band of the camera. Combining these bands using different mathematical equations results in different values. These values can be represented by different colors (Fig.8.). Numerical values of the indices can be exported in a Microsoft Excel file.

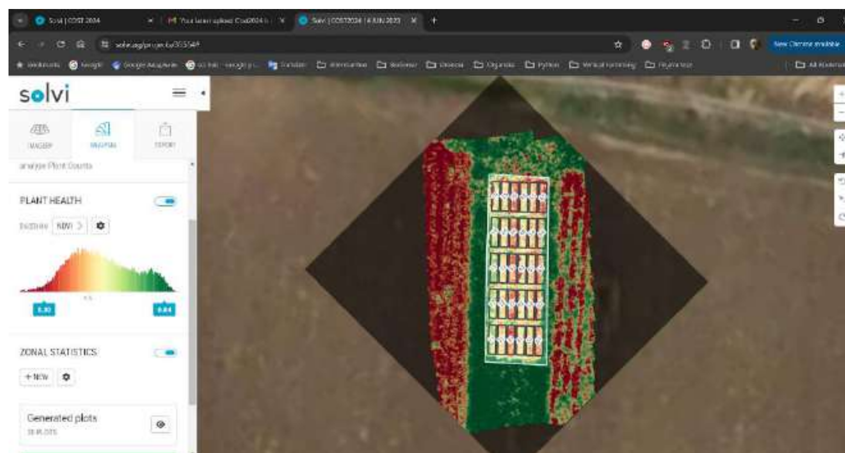

Fig. 8. Analizing of different VI values in Solvi web app

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- Correlating these values with various numerically represented plan characteristics, such as yield components, the presence of different chemical components, or the effects of experimental treatments (e.g., different fertilization or irrigation dosages, different genotypes), can determine the linkage and correlation between vegetation indices and different factors. Collecting big data regarding the plant's condition in the field and VI values can be used to make predictions and create scenarious based on the pictures captured by a multispectral camera on a drone.

- In summary, the use of UAVs equipped with multispectral cameras for detecting differences in cereal crops with various sulfur treatments represents a powerful application of remote sensing technology in precision agriculture, enabling farmers and researchers to make informed decisions to enhance crop production and sustainability.

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ACRYRED • COST Action CA21149 • Reducing acrylamide exposure of consumers by a cereal supply-chain approach targeting asparagine

TRAINING SCHOOL
Genetics and Agronomy for Low Acrylamide-Forming Potential in Cereals

WG2 Leaders: Elena Baldoni (IT), Viktor Korzun (DE)
Local organizer: Slađana Žilić (SRB)

Date: May 22 – 24, 2024
Hosting Institution: Maize Research Institute, Zemun Polje (MRIZP) mrizp
Location: Belgrade-Zemun, Serbia

Number of Trainees: 22 from 10 countries
(Italy, Germany, Spain, Slovakia, Poland, Ireland, United Kingdom, Netherlands, Turkey, Serbia)
Number of Trainers/Invited lecturers: 6 foreign + 6 Serbian
Number of Trainers/Practical training: 9 Serbian

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